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TITLE: Tumor-Mediated Suppression of Dendritic Cell Vaccines

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13. ABSTRACT (Maximum 200 Words) The unique ability of dendritic cells to potentially stimulate naïve lymphocytes in an antigen-specific fashion has made them prime candidates for cancer immunotherapy. A number of tumor-derived products have been suggested to promote tumor establishment and progression by interfering with numerous DC functions required for the induction of a potent antitumor response. One of the best characterized of these tumor-derived factors is Transforming growth factor-beta (TGF-β), a multifunctional cytokine that exerts potent suppressive effects on cells of the immune system. TGF-β specifically interferes with DC maturation, chemotaxis, antigen recognition and T cell activation. These findings strongly suggest that a strategy that protects DCs from the harmful effects of TGF-β should enhance the effectiveness of DC-based vaccines. The goal of this study is to protect DCs from the direct actions of tumor-derived TGF-β in order to improve their effectiveness as cancer vaccines. The hypothesis to be tested is that interference with TGF-β signal transduction in DCs will abrogate tumor-derived TGF-β-mediated immunosuppression leading to more effective DC vaccines. The specific aims of this study are to: 1) determine the effect of tumor-derived TGF-β on antigen presentation and in vivo migration of DCs. 2) evaluate the impact of tumor-derived TGF-β on DC vaccines. 3) protect DCs from TGF-β-mediated immunosuppression by blocking TGF-β signal transduction.				
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Key accomplishments

- 1. Determination of the effects of TGF- β on dendritic cell expression of surface markers.**
- 2. Evaluation of the effects of TGF- β on the ability of dendritic cells to present antigen.**
- 3. Evaluation of the effects of TGF- β on in vivo migration of dendritic cells.**
- 4. Determination of the effect of TGF- β on the efficacy of dendritic cell vaccines.**
- 5. Generation and evaluation of Smad7 adenovirus.**

Determination of the effects of TGF- β on dendritic cell expression of surface markers

The presence of tumor-derived TGF- β in the tumor microenvironment suggests that dendritic cells recruited to the tumor site must perform their critical functions amidst high levels of TGF- β . The effect of short-term (48 hour) and chronic (6 days) TGF- β exposure on expression of characteristic cell surface markers was evaluated. Short-term exposure did not alter the expression of the surface molecules; CD11c, I-A^d, B7.1, B7.2 or CD40 (Figure 1A). Chronic exposure to TGF- β resulted in decreased expression of I-A^d, B7.1 and CD40 (Figure 1B). Exposure to TGF- β reduces the expression of surface molecules critical for antigen presentation to and T cell activation.

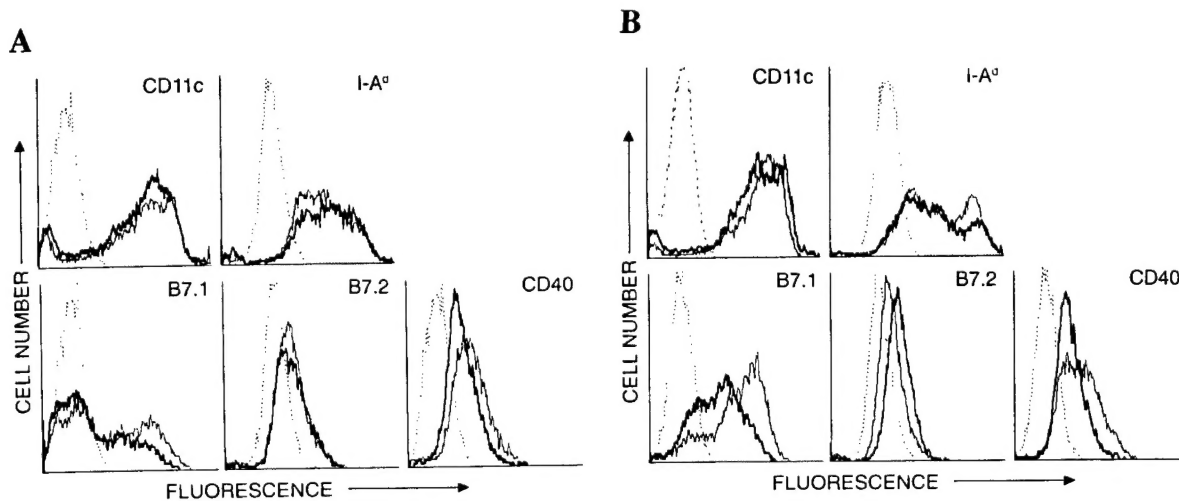


Figure 1. Chronic exposure to TGF- β modulates expression of critical co-stimulatory molecules. Day 6 dendritic cells received short-term (48 hour) exposure (A) or chronic (6 day) exposure (B) to 10ng/ml TGF- β 1. Cytokines were replenished every two days. Following exposure to TGF- β , DC were collected and stained with anti-CD11c, anti-I-A^d, anti-B7.1, anti-B7.2 and anti-CD40 antibodies and analyzed by flow cytometry. Data represents mean \pm SEM of three independent experiments. Broken line represents unlabeled cells control, thin line represents untreated cells, bold line represents cells treated with TGF- β .

Evaluation of the effects of TGF- β on the ability of dendritic cells to present antigen

To evaluate the effect of TGF- β exposure on the antigen presenting functions of DC, allogeneic mixed lymphocyte reactions were performed using dendritic cells generated from Balb/c mice and lymphocytes from C57/BL6 mice. The ability of DC to stimulate allogeneic T lymphocytes was decreased following chronic exposure to TGF- β (Figure 2A). Short-term exposure to TGF- β did not affect the allo-stimulatory capabilities of dendritic cells (Figure 2B).

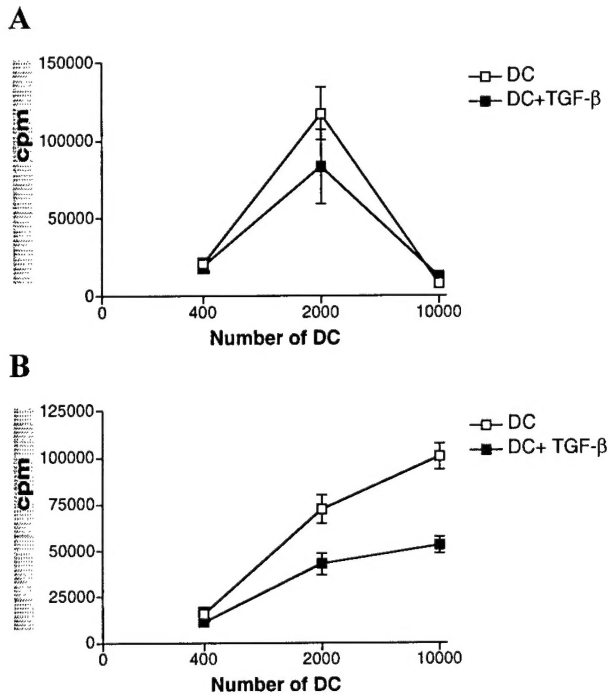


Figure 2. Chronic exposure to TGF- β inhibits DC allostimulatory capacity. DC received short-term (A) or chronic (B) exposure to TGF- β as indicated previously. Various numbers of DC were incubated with 2×10^5 splenic T cells isolated from C57/BL6 mice for 5 days with the addition of $1 \mu\text{Ci}$ of [^3H]thymidine for the last 18 hours of culture. Values represent mean \pm SEM of six replicates. Data are representative of three independent experiments.

The ability of dendritic cells to stimulate naive T cells was evaluated. Dendritic cells were evaluated for presentation of OVA peptide to naive T cells from DO11.10 TCR-transgenic mice. DO11.10 T cells express a transgenic T cell receptor that recognizes OVA peptide fragment 323-339 (KISQAVHAAHAEINEAG) in the context of I-A^d. Chronic TGF- β exposure reduced the ability of dendritic cells to stimulate proliferation of OVA specific T cells (Figure 2A). Short-term TGF- β exposure was not sufficient to affect proliferation of OVA transgenic T cells (Figure 2B). It is apparent that chronic exposure to TGF- β reduces the antigen presentation and lymphocyte stimulatory abilities of dendritic cells.

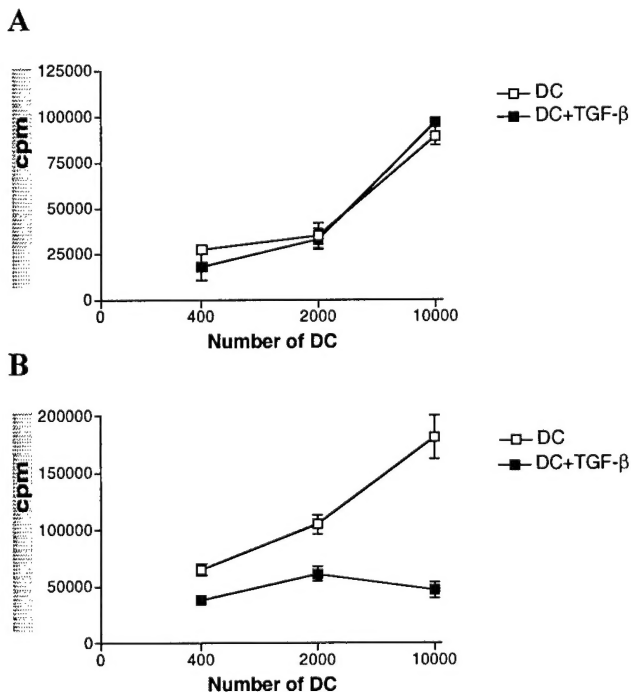


Figure 3. Chronic exposure to TGF- β inhibits DC ability to present OVA peptide. DC received chronic (A) or short-term exposure (B) to TGF- β as previously indicated. Various numbers of DC were incubated with 2×10^5 splenic T cells isolated from Balb/c-TgN (DO11.10) 10 Loh mice in the presence of $0.1 \mu\text{g/ml}$ of OVA peptide for 5 days with the addition of $1 \mu\text{Ci}$ of [^3H]thymidine for the last 18 hours of culture. Values represent mean \pm SEM of four replicates. Data are representative of two independent experiments.

Effect of TGF- β on in vivo DC migration

Following antigen uptake at the tumor site it is necessary for the dendritic cell to migrate to secondary lymphoid organs to prime tumor-specific T lymphocytes. The ability of dendritic cells to migrate from the periphery to draining lymph nodes following TGF- β exposure was evaluated. Dendritic cells were labeled with PKH-67, a green fluorescent dye, and injected subcutaneously into the hind flank of mice. Forty-eight hours following injection, draining inguinal lymph nodes were harvested. Migrated dendritic cells were enumerated by scanning laser cytometry of cytospin preparations of lymph node cell suspensions. Immature dendritic cells were less migratory than TNF- α -treated mature DC. Mature DC were 2-3 times more migratory toward the draining lymph node than immature DC. Treatment with TGF- β resulted in a 36% decrease in DC migration to the draining lymph nodes (Table 1).

Cells	Total cells scanned	% DC	DC /10 ⁶ LN cells
iDC	15829	0.032 (5)	316
iDC + TGF- β	18932	0.032 (6)	317
mDC	33430	0.114 (38)	1137
mDC + TGF- β	35745	0.073 (26)	727

Table 1. Exposure to TGF- β diminishes DC ability to migrate to the lymph nodes. DC were cultured in the absence (iDC) or presence (mDC) of TNF- α with or without the addition of 10 ng/ml of TGF- β for 48 hours. DC were then labeled with PKH-67 membrane dye and eight million cells were injected subcutaneously into each of two Balb/c mice per group. Forty-eight hours following injection, inguinal lymph nodes were harvested from mice and disaggregated. Lymph node cells were fixed and stained with Propidium iodide/RNase I solution and analyzed by laser scanning cytometry.

Determination of the effect of tumor-derived TGF- β on the efficacy of dendritic cell vaccines

In order to reduce the amount of TGF- β present in the tumor microenvironment the TGF- β neutralizing antibody, 2G7 was administered both systemically and intratumorally (i.t.) in combination with i.t. injected tumor lysate pulsed, matured dendritic cells. Treatment of established 4T1 primary tumors with dendritic cell vaccination in combination with 2G7 significantly reduced tumor progression as compared to untreated tumors. Treatment of tumors with dendritic cell alone, 2G7 antibody alone, or isotype antibody alone did not significantly reduce tumor volume (Figure 3). DC vaccination of 4T1 tumors transduced with the antisense TGF- β transgene (4T1-asT) and mock-transduced 4T1 (4T1-N) were performed to evaluate the impact of TGF- β production on vaccine efficacy. TGF- β production by 4T1-asT cells is suppressed by 93% (0.083 ± 0.003 ng/ml) compared to 4T1-N cells (1.244 ± 0.188 ng/ml). Treatment with DC and 2G7 antibody significantly inhibited tumor growth in mice bearing established 4T1-asT primary tumors as compared to mice bearing established 4T1-N tumors. Additionally 40% of the mice bearing 4T1-asT tumors treated with DC and 2G7 antibody demonstrated complete tumor regression as compared to only 20% of mice bearing 4T1-

N tumors (Figure 4). These data suggest that tumor-derived TGF- β prevents DC from generating an effective anti-tumor immune response.

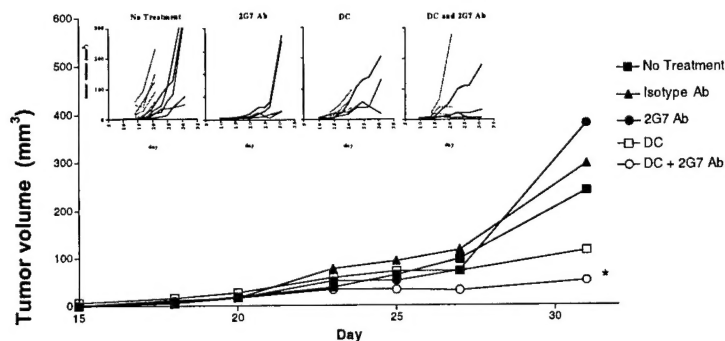


Figure 3. TGF- β neutralizing antibody enhances the ability of DCs to inhibit tumor growth. Balb/c mice with established 4T1 tumors were vaccinated intratumorally on day 15, 20 and 25 with 1.5×10^6 tumor lysate-pulsed DCs alone or in combination with 200 μ g i.t. and 200 μ g i.p. of 2G7 antibody. Control mice were treated with 2G7 or isotype antibody alone. Mice were monitored for tumor growth. Tumor volume was calculated as $(l \times w^2)/2$. Data represents mean tumor volume of 4 or 5 mice. * Mice treated with DC + 2G7 antibody exhibited significantly decreased tumor volumes as compared to untreated mice ($p=0.032$) on day 32. Inlay graphs represent tumor growth of individual mice.

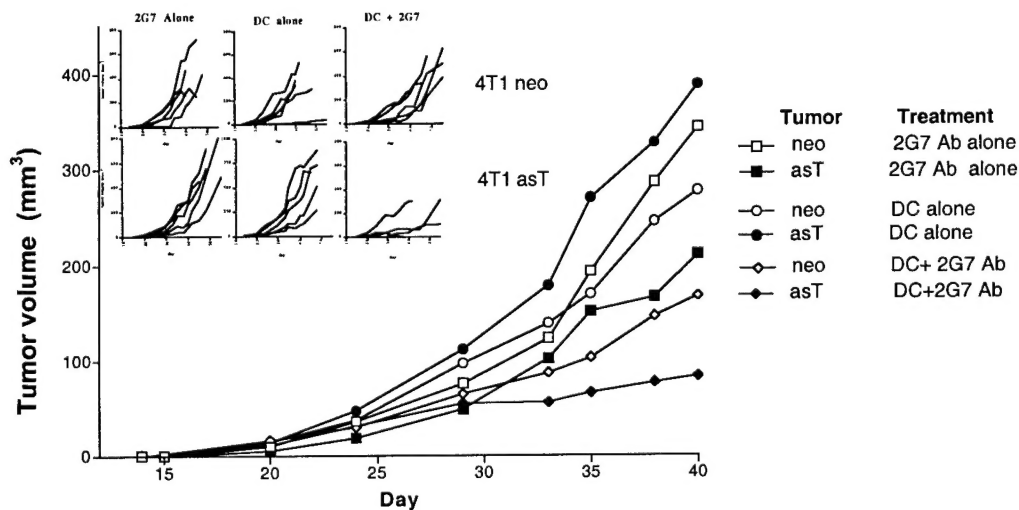


Figure 4. Tumor derived TGF- β suppresses the ability of DC to inhibit tumor growth. Balb/c mice with established 4T1-N (mock-transfected) and 4T1-asT (transfected with antisense TGF- β gene) tumors were vaccinated intratumorally on day 15, 20, and 25 with 1.5×10^6 tumor lysate-pulsed mature DCs alone or in combination with 200 μ g i.t and 200 μ g i.p. of 2G7, TGF- β neutralizing antibody. Control mice were treated with 2G7 antibody alone. Mice were monitored for tumor growth. Data represent mean tumor volume of 5 mice. Insert graphs represent tumor growth of individual mice.

Expression of Smad7 by an Adenovirus Vector

To overcome the immunosuppressive effects of TGF- β we have constructed an adenoviral vector that contains the Smad7 gene linked to the FLAG fusion protein (AdSmad7) (Figure 5). Smad7 is an inhibitor of TGF- β signaling. We have further evaluated the ability of this adenoviral vector to express recombinant Smad7 protein in infected cells. 4T1 cells were infected with AdSmad7 and analyzed by immunofluorescence for FLAG protein expression. 4T1 cells transduced with AdSmad7 expressed the FLAG epitope while non-transduced cells did not (Figure 6). In future experiments DC will be infected with AdSmad7 and evaluated for decreased responsiveness to TGF- β .

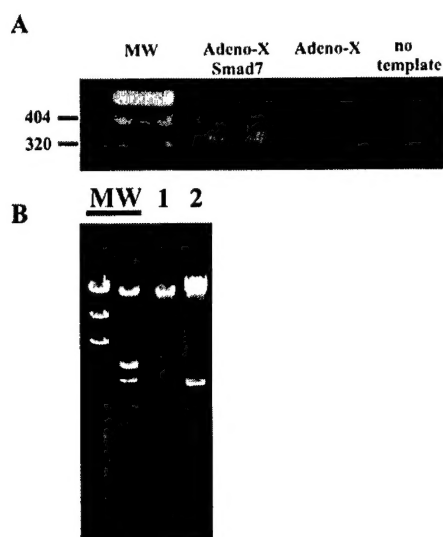


Figure 5. Cloning of Smad7 into Adeno-X viral DNA.

A FLAG-tagged mouse Smad7 gene was excised from pcDNA-3 and cloned sequentially into the Nhe I and Xba I sites of the pShuttle plasmid and the I-Ceu I and PI-Sce I sites of the Adeno-X Viral DNA. **A.** The Smad7 gene was amplified from the Smad7-Adeno-X vector by PCR. **B.** A restriction digest of Adeno-X viral DNA (lane 1) and the Smad7-Adeno-X vector (lane 2) was performed with I-Ceu I and PI-Sce I to yield a 32kb band representing the Adeno-X viral DNA and a 4kb band representing the Smad7 insert.

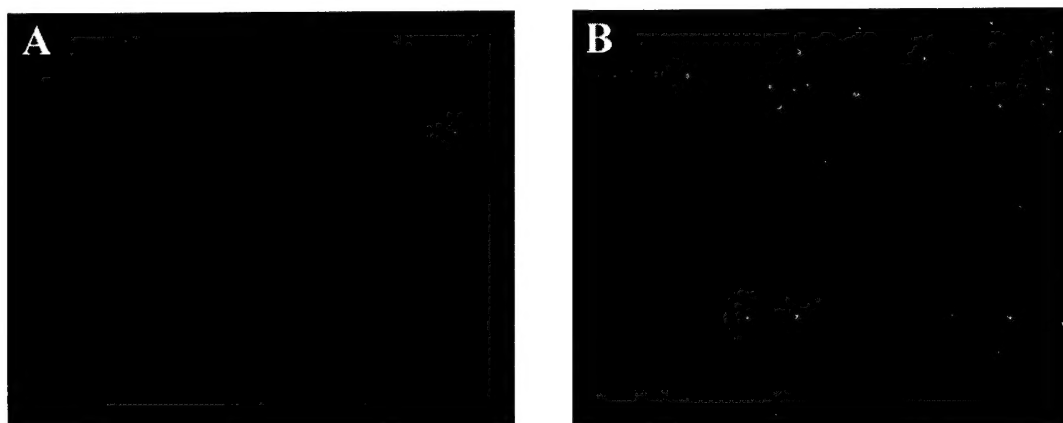


Figure 6. Expression of Smad7 by 4T1 cells. 4T1 cells were infected with AdSmad7, 72 hours later, cells were fixed and immunostained with anti-FLAG M2 FITC- conjugated antibody (Sigma) and analyzed for immunofluorescence by confocal microscopy. **(A)**, non-transduced cells stained with anti-FLAG antibody, **(B)**, AdSmad7-transduced cells stained with anti-FLAG antibody. Magnification = 20X.

Reportable Outcomes

Funding applied for and received based on research supported by this grant

National Cancer Institute

Grant # 1RO1CA9411-01

“Effect of tumor-derived TGF- β on dendritic cell vaccines”

Presentations

Kobie, J.J., Whitesell, L.J., and Akporiaye, E.T. Effects of transforming growth factor - β on the ability of dendritic cells to stimulate an anti-tumor response. Poster. Annual meeting of the Society for Biological Therapy, Washington, DC, November 16-19, 2001.

Kobie, J.J., Lou, S., Whitesell, L.J., and Akporiaye, E.T. Transforming growth factor - β diminishes the ability of dendritic cells to stimulate an antitumor immune response. Annual Meeting for the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002.

Akporiaye, E.T., Kobie, J.J., and Whitesell, L.J. Transforming growth factor- β inhibits in vivo migration and T cell priming by tumor lysate-pulsed dendritic cells. International Cancer Congress of the International Union against Cancer, Oslo, Norway, June 30 – July 5, 2002.

Conclusions

TGF- β interferes with critical functions of dendritic cells, which are necessary for developing an effective anti-tumor immune response. The ability of dendritic cells to present antigen is diminished by chronic TGF- β exposure. Migration of dendritic cells from the periphery to secondary lymphoid organs is also inhibited by exposure to TGF- β . Most importantly, tumor-derived TGF- β suppresses the ability of DC to mediate regression of established tumors. Together these results suggest that DC that are rendered unresponsive to TGF- β are likely to be more effective at mediating tumor regression. By demonstrating the immunosuppressive effects of TGF- β on DC we have identified a potential target for improvement of DC – based cancer vaccines.